(12) UK Patent Application (19) GB (11) 2 196 348 (13) A

(43) Application published 27 Apr 1988

(21) Application No 8722473

(22) Date of filing 24 Sep 1987

(30) Priority data
(31) 7158 (32) 3 Oct 1986 (33) CS

(71) Applicant
Ceskoslovenska Akadmie Ved

(Incorporated in Czechoslovakia)

Praha, Czechoslovakia

(72) Inventors
Jan Kovar
Frantisek Franek

(74) Agent and/or Address for Service
Matthews Haddan & Co.,
33 Elmfield Road, Bromley, Kent BR1 1SU

(51) INT CL⁴
C12N 5/02 5/00

(52) Domestic classification (Edition J):
C6F HC1
U1S 1068 2419 C6F

(56) Documents cited
Biotechnol Lett. (1987) Vol 9(4) p259–64

(58) Field of search
C6F
Selected US specifications from IPC sub-class C12N

(54) Synthetic medium for hybridoma and myeloma cell cultivation

(57) Synthetic medium for cultivation of hybridoma and myeloma cells comprising anorganic salts, monosaccharides, amino acids, vitamins, substances buffering changes of pH, possibly also sodium pyruvate, antibiotics, indicators of pH and a supplement formed by at least one water soluble iron compound in a concentration of 2×10^{-5} to 1×10^{-2} mol/l.

The effectiveness of the supplement can be increased by the presence of compounds biogenic trace elements, ascorbic acid, steroid substances and of amines.

The iron compound is preferably, iron sulphate, ferric citrate or chloride or potassium ferricyanide.

The medium may also contain, hydrocortisone, zinc, selenium or ethanolamine.

GB 2 196 348 A

SPECIFICATION

Synthetic medium for hybridoma and myeloma cell cultivation

The invention relates to a synthetic medium for hybridoma and myeloma cell cultivation.

Hybridomas obtained by somatic hybridization of a myeloma cell and of a lymphocyte synthetizing an antibody are industrially used for manufacture of monoclonal antibodies. Myeloma cells, into which by methods of genetic engineering a gene of a biologically active protein is introduced, for instance a gene of an enzyme, an enzyme inhibitor, a hormone, an immunoregulator, in order to manufacture said proteins, are equally industrially employed. The increasing need of different monoclonal antibodies, particularly for therapeutic purposes and for immunoaffinity chromatography, where kilogram quantities are required, equally as the need of different regulating proteins for therapeutic purposes, lead to an endeavour to look for more effective and economically more advantageous manufacturing methods of said materials.

Hybridoma and myeloma cells are actually cultivated by first multiplying in laboratory vessels such as plastic dishes and bottles, the final cultivation and production of the protein is accomplished in an industrial fermentor. The monoclonal antibody or some other protein secreted into the cultivation medium is obtained from the culture medium after centrifugation or filtration of the cells. The isolation of the produced protein is the more difficult, the more protein the original culture medium has contained. The concentration of the produced protein, for instance of the monoclonal antibody in the cultivation medium is relatively low.

The synthetic culture medium contains basic culture components and a supplement. Basic culture components are anorganic salts, for instance sodium chloride and sodium phosphate, monosaccharides, for instance glucose or galactose, amino acids, for instance tryptophan, methionine, threonine and glutamine, vitamines, for instance thiamine and nicotinamine, substances buffering changes of pH, for instance H-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid, possibly sodium pyruvate, antibiotics and an indicator of changes of pH. These basic cultivation components are insufficients for hybridoma and myeloma cells, the same as for the animal cells and are therefore supplemented by 10% by volume of a blood serum, for instance cattle-, horse- or fetal bovine serum. The blood serum introduces into the cultivation medium unknown substances, some of which are supporting the growth of cells, others can inhibit the growth. It is therefore necessary to test the blood serum prior to its application and select suitable lots. Thus the costs for manufacture of the culture medium are increasing. A number of recipes for so-called serum-free culture media have been proposed where the blood serum is replaced by several defined proteins, mostly by transferrin, serum albumin and insulin, possibly by other materials such as unsaturated fatty acids, amines and low molecular hormones (see J.Kovář, F. Franěk: Methods in Enzymology 121: 277,1986).

All proteins actually added into culture media for cultivation of hybridoma and myeloma cells have to be free of toxic admixtures, which increases the economic claims on manufacture. Another drawback of existing cultivation media is the difficult isolation of products of the life activity of hybridoma and myeloma cells from culture media, which separation is accomplished by repeated separation of required products of protein character from proteins contained in the culture medium in a large excess.

The mentioned drawbacks are eliminated by application of a culture medium according to this invention which comprises in addition to basic components as are anorganic salts in a concentration of 5000 to 12000 mg/l, monosaccharides in a concentration of 300 to 5000 mg/1, amino acids in a concentration of 200 to 5000 mg/1, vitamines in a concentration of 2 to 100 mg/1, substances buffering changes of pH in a concentration of 200 to 20000 mg/l, possibly sodium pyruvate, antibiotics, an indicator of changes of pH, also a supplement formed by at least one water soluble iron compound in a concentration 2×10^{-5} to 1×10^{-2} mol/l of culture medium, advantageously ferric citrate, iron sulphate, ferric chloride or potassium ferricyanide.

It is advantageous in case the culture medium contains in combination with the supplement compounds of biogenic trace elements, particularly of selenium and zinc, ascorbic acid, steroid substances particularly cortisone and amines, advantageously ethanolamine, the presence of which increases the effectiveness of the supplement.

It has been found, that an entirely indispensable protein component of the serum-free culture medium is transferrin (see J.Kovář, F. Franěk: Methods in Enzymology, 121: 277,1986). As transferrin acts as a transport substance of iron from the surrounding medium into the cell, its functionning could be substituted by the presence of water soluble iron compounds of a higher concentration than have been up to now applied in cultivation media. A number of authors (for instance P.D. Phillips, V.J. Cristofalo in Exp. Cell Res., 134:297, 1981 V. Perez.Infante, J.P. Mather in Exp. Cell Res., 142: 325, 1982; R. Taetle, K. Rhyner, J. Castagnola, D.To, J. Mendelsohn, J.Clin, Invest., 75:1051,1985) have used instead of transferrin water soluble iron compounds in concentration of 1×10^{-6} to 20×10^{-6} mol/l. These concentrations are

however insufficient in case of hybridoma and myeloma cells. It has been found that iron compounds up to a concentration of 1×10^{-2} mol/l are not toxic for hybridoma and myeloma cells and on the other side concentrations of iron compounds higher than 2×10^{-5} mol/l enable growth of hybridoma and myeloma cells in a protein-free culture medium. Iron compounds capable to replace transferrin are for instance ferric citrate, iron sulphate, ferric chloride and potassium ferricyanide. Effective concentrations are within limits of 2×10^{-5} up to 1×10^{-2} mol/l.

Advantages of the cultivation medium according to this invention is the easy isolation of the protein separated by cells, for instance of monoclonal antibodies, as the protein secreted by cells is the sole protein which is after cultivation present in the culture medium.

The advantages of the culture medium according to this invention will be more clearly shown on hand of following examples.

Example 1

A basic culture medium which in addition to the medium RPMI 1640 contained L-glutamine /300 ug/ml, sodium pyruvate/110 ug/ml/, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid/$1,5 \times 10^{-2}$ mol/l, penicillin/100 units/ml/, streptomycin/100 ug/ml/ and gentamycin/40 ug/ml/ has been used in order to prepare a chemically defined protein-free cultivation medium. The protein-free cultivation medium contained thereafter as substituted of a serum the following additional materials: ethanolamine /2×10^{-5} mol/l/, ascorbic acid /2×10^{-5} mol/l/, hydrocortisone /5×10^{-9} mol/l/, cadmium sulphate /$CdSO_4.8/3\ H_2O$, 5×10^{-8} mol/l, cobalt chloride, /$CoCl_2.6\ H_2O$, 1×10^{-8} mol/l, copper sulphate ($CuSO_4.5\ H_2O$, 1×10^{-8} mol/l, ammonium molybdate /$NH_4\ _6Mo_7O_{24}.4\ H_2O$, 5.10^{-10} mol/l, manganese dichloride /$MnCl_2.4\ H_2O$, 5×10^{-10} mol/l, nickel sulphate /$NiSO_4.6\ H_2O$, $2,5 \times 10^{-10}$ mol/l, sodium selenite/ /Na_2SeO_3, 4×10^{-8} mol/l, sodium silicate (Na_2SiO_3, 2×10^{-7} mol/l, stannous chloride /$SnCl_2.2\ H_2O$, $2,5 \times 10^{-10}$ mol/l, ammonium vanadate / NH_4VO_3, $2,5 \times 10^{-0}$ mol/l, zinc sulphate /$ZnSO_4.7\ H_2O$, 1×10^{-6} mol/l and ferric citrate, 5×10^{-4} mol/l.

Into 6 ml of the protein-free culture medium of the mentioned composition in a Petri dish (diameter 6 cm) cells of the hybridoma PLV-01 have been inocculated in such an amount that the density of cells has been 50×10^3 cells/ml. After 4 days of cultivation in a thermostat in a humidifed atmosphere of 5% by volume of carbon dioxide in air at a temperature of 37°C, the cells have multiplied to a density of $1,53 \times 10^6$ cells/ml. The number of cells has thus in the course of cultivation for 4 days multiplied approximately thirtytimes.

By a similar test with cells of the hybridoma of $1,36 \times 10^6$ cells/ml within 4 days. The number of cells has at this test after 4 days of cultivation increased fitytimes.

Cells of myeloma FO and of hybridomas PLV-01, CMH-02 and T3-03 have been separately inocculated to volumes of 1 ml of a protein-free culture medium of the mentioned composition contained in wells of a plastic multi-dish with 24 wells. After the first day of cultivation at mentioned conditions the number of cells in a well has been determined and after next 24 hours this number has been checked again. Thus the mean doubling-time in the exponential growth phase for each hybridoma or myeloma has been determined. The mean doubling-time for the myeloma FO has been found to be 15,6 h, for the hybridoma PLV-01 14,8 h, for the hybridoma CMH-02 14,6 h and for the hybridoma T3-03 11,8 h.

Cells of the hybridoma PLV-01 and of the hybridoma PGG-05 have been inocculated separately in a number of 60×10^3 cells to volumes of 1 ml of a protein-free culture medium of the mentioned composition situated in wells of a plastic multi-dish with 24 wells. For comparison cells of the same hybridomas have been inocculated into a serum-free culture medium containing 5 mg/l of trasnferrin and 10 mg/l of insulin / SFH medium without lineloic acid and albumin, see: J.Kovář and F. Franěk: Methods in Enzymology, 121: 277, 1986/. After 4 days of cultivation at above mentioned conditions the number of cells and the concentration of monoclonal antibodies in the culture medium have been determined using an enzymoimmunologic test based on the determination of the peroxidase activity. In case of a growth of the hybridoma PLV-01 in the protein-free medium from an inocculum of 60×10^3 cells, the concentration of the monoclonal antibody expressed in relative units of absorbance had the value of A_{490}=0,298. In the comparative experiment carried out in an SFH medium values of 706×10^3 cells and A_{490}=0,310 have been obtained. In case of a growth of the hybridoma PGG-05 in a protein-free culture medium for 675×10^3 cells, the concentration of the monoclonal antibody expressed in relative units of absorbance had the value of A_{490}=1,32. In the comparative experiment carried out in an SFH medium values of 731×10^3 cells and A_{490}=1,26 have been obtained.

Example 2

Cells of a hybridoma PLV-01 have been inocculated in a number of 10×10^3 cells into 0,1 ml of a protein-free cultivation medium of the composition as in example 1 placed in wells of a plastic cultivation microplate with 96 wells. For comparison purposes cells of this hybridoma have been inocculated into a

placed the ferric citrate. After 3 days of cultivation at conditions as in example 1 the hybridoma cells have multiplied to 131×10^3 cells in the culture medium with ferric citrate and to 82×10^3 cells in the cultivation medium with ferric chloride.

Example 3

Cells of the hybridoma PLV-01 have been inocculated in an amount of 10×10^3 cells to 0,1 ml of basic components mentioned in example 1. As supplement 5×10^{-4} mol/l of potassium ferricyanide have been added into the culture medium. After 3 days of cultivation at the temperature of 37°C and of 4,5% per volume of carbon dioxide in a humidified atmosphere at pH 7,4 of the medium the cells have multiplied to 62×10^3.

Example 4

Cells of the hybridoma PLV-01 have been inocculated in an amount of 10×10^3 cells into 0,1 ml of basic components mentioned in example 1. Iron sulphate in a concentration of 1×10^{-4} mol/l has been added as supplement into the culture medium. After 3 days of cultivation at a temperature of 37°C and 4.5% per volume of carbon dioxide in a humidified atmosphere at pH 7,4 of the medium the cells have multiplied to 25×10^3.

Example 5

Cells of the hybridoma PLV-01 have been inocculated in an amount of 10×10^3 into 0,1 ml of protein-free culture medium of the composition as in example 1 placed in wells of a plastic cultivation microplate with 96 wells. For comparison cells of this hybridoma have been inocculated into a protein-free culture medium containing in addition to the basic medium as in example 1 solely ferric citrate (5×10^{-4} mol/l). After 3 days of cultivation at conditions as in example 1 the hybridoma cells have multiplied to 118×10^3 cells in the protein-free culture medium and to 103×10^3 cells in the protein-free culture medium where solely ferric citrate has been added.

CLAIMS

1. Synthetic medium for hybridoma and myeloma cell cultivation comprising anorganic salts in a concentration of 5000 to 12000 mg/l, monosaccharides in a concentration of 300 to 5000 mg/l, amino acids in a concentration of 200 to 5000 mg/l, vitamins in a concentration of 2 to 100 mg/l, substances buffering changes of pH in a concentration of 200 to 20000 mg/l, possibly also sodium pyruvate, antibiotics, an indicator of pH changes and a supplement, characterised in that said supplement is formed by at least one water soluble iron compound in a concentration of 2×10^{-5} to 1×10^{-2} mol/l.

2. Synthetic medium as in claim 1, characterised in that the water soluble iron compound is iron sulphate, ferric citrate, ferric chloride, potassium ferricyanide.

3. Synthetic medium as in claim 1 or 2 characterised in that it contains compounds of biogenic trace elements, advantageously selenium, zinc in a concentration of 0,002 to 2 mg/l.

4. Synthetic medium as in one of claims 1 to 3 characterised in that it contains ascorbic acid in a concentration of 1 to 100 mg/l.

5. Synthetic medium as in one of claims 1 to 4 characterised in that it contains steroid substances, advantageously hydrocortisone in a concentration of 0,0001 to 0,1 mg/l.

6. Synthetic medium as in one of claims 1 to 5 characterised in that it contains amines, advantageously ethanolamine in a concentration of 0.1 to 15 mg/l.

7. Synthetic medium for hybridoma and myeloma cell cultivation as claimed in Claim 1 substantially as described in any one of the examples disclosed herein.

Published 1988 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office, Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Burgess & Son (Abingdon) Ltd. Con. 1/87.